added to the medium together with 1-C^{14}-acetate offer little evidence to support the squalene-precursor hypothesis. It is true that squalene does lower the conversion of acetate to sterols, but it simultaneously affects the conversion of acetate to fatty acids. This may be explained by considering the formation of a common precursor to both sterols and fatty acids from oxidation of squalene. It should be realized too, that only when large, unphysiological amounts of squalene are added, can a marked reduction in acetate conversion be observed. A mere 50% reduction is no indication that a compound is an intermediate to the sterols, but that it is rather an equal competitor with acetate. A direct precursor should be utilized much more readily, although solubility differences between acetate and squalene may play some role in these results.

The evidence obtained from cell-free yeast extract experiments, especially when considered with results from whole yeast cells, lends no support to the hypothesis that squalene is an obligatory intermediate to the yeast sterols. It indicates, on the contrary, that squalene in yeast may be metabolized along different lines although the possibility that squalene and the sterols stem from the same precursors is not excluded.

Acknowledgment.—The authors are grateful to Dr. Adrian Kuyper for guidance in the radioactive tracer work, to the Red Star Yeast Company for supplying them with fresh yeast throughout the course of the experiments, and to the American Medical Association for a grant-in-aid to complete their work.

DETROIT, MICHIGAN

[CONTRIBUTION FROM THE DEPARTMENTS OF BOTANY AND BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Isolation, Structure and Synthesis of Kinetin, a Substance Promoting Cell Division[1,2]

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A substance which markedly promotes cell division in various plant tissue cultures in concentrations as low as one microgram per liter has been isolated in pure form from heated deoxyribonucleic acid preparations, and has been shown by degradation and synthesis to be 6-furfurylaminopurine. The specific name *kinetin* has been applied to this substance, and the generic term *kinin* is suggested for any substance which similarly stimulates cytokinesis.

Attempts in these laboratories to isolate from coconuts a factor promoting cell division in plant tissues resulted in some 4000-fold concentration of the active substance(s), but no isolation of any active pure compound was accomplished.[3,4] In related growth studies, it was observed that dried brewer's yeast similarly promoted cell division and that a small portion of the active material was extractable from aqueous solutions by *ether*. It seemed likely that this ether-soluble factor would be simpler in chemical composition and more readily purified than the highly water-soluble, ether-insoluble material in coconut concentrates.[3] A preliminary work-up of a yeast ether extract did, in fact, lead rather easily to high potency material. Activity was correlated with a substance which had a maximum absorption at 268 mμ and was precipitated from acid solutions by silver nitrate. This material was obtained in low yield, however, so other possible sources were investigated.

Since the active substance had purine-like properties, other purine-containing materials were tested, and eventually an old sample of deoxyribonucleic acid (DNA) was located which was extraordinarily potent. Its ether extract, without further purification, equalled the activity of the best concentrates previously obtained in our laboratories from any source. Fresh DNA samples were inactive, but could be activated by slurrying in water and autoclaving. Relatively large amounts of rich concentrates thus became available and, on further purification, mainly by ion-exchange chromatography, readily yielded a very highly active crystalline product, I.

The newly isolated compound proved to have the empirical formula $C_{10}H_9N_5O$. It was amphoteric (pK_a values approximately 4 and 10) and optically inactive. It dissolved easily in aqueous strong acids and alkalies and in glacial acetic acid, was slightly soluble in ethanol, butanol, acetone and ether, but practically insoluble in distilled water. It sublimed unchanged at 220° at atmospheric pressure and was unaffected by autoclaving either at *p*H 0.5 or 12.0. However, autoclaving in stronger acid solution, *e.g.*, 2.0 *N* sulfuric acid, did lead to loss in physiological activity.

The high nitrogen content, ultraviolet spectrum (single band near 268 mμ), amphoteric character, precipitation with silver, and isolation from DNA all suggested that I might be a purine derivative. The solubility in organic solvents pointed to a relatively non-polar grouping in the molecule. First guesses as to the nature of I, therefore, centered around dehydrated nucleosides. This hypothesis was strengthened when I was subjected to strong acid hydrolysis and the hydrolysate chromatographed on paper with solvent systems designed for purine separation. The original ultraviolet quenching spot characteristic of I disappeared after hydrolysis, and was replaced by another having the R_F value and, after elution, the ultraviolet spectrum of adenine. The presence of adenine in the hydrolysate was then confirmed by isolation of the

(1) Preliminary reports of this work have appeared in (a) THIS JOURNAL, **77**, 1392 (1955); (b) **77**, 2662 (1955).

(2) This work was in part supported by grants from the American Cancer Society, The National Science Foundation and the Wisconsin Alumni Research Foundation.

(3) J. R. Mauney, W. S. Hillman, C. O. Miller, F. Skoog, R. A. Clayton and F. M. Strong, *Physiol. Plantarum*, **5**, 485 (1952).

(4) D. A. Buyske, Ph.D. dissertation, University of Wisconsin, 1954.

crystalline picrate and by chromatographic analysis on an ion-exchange column according to the method of Wall.[5] All of the nitrogen and one-half of the carbon atoms of I were thus accounted for as adenine.

If the remaining five carbons were derived from dehydration of a pentose,[6] it was expected that strong acid treatment of I might yield levulinic acid, and in fact the odor of levulinic acid was evident in the sulfuric acid hydrolysate. Efforts to prepare crystalline derivatives failed, but comparative paper chromatograms of the crude dinitrophenylhydrazone and an authentic sample gave strong indications that levulinic acid was actually present.

This result made it very probable that the five unidentified carbons were linked together to form a single side group on the adenine moiety. Further evidence in this direction was that I gave with cysteine and 70% sulfuric acid the Dische color reaction[7] characteristic of deoxyribosides, although the amount of color developed under standard conditions[8] was only 12% of that produced by an equimolar amount of adenine deoxyriboside. As to the exact nature of the side group, however, little additional direct evidence was uncovered except that the infrared absorption band at 8 μ indicated an ether linkage.

Attractive possibilities were that the group comprised a furan nucleus plus one extra carbon present either as a side methyl on the furan ring or as a methylene radical between the furan and purine nuclei. Each of these possibilities accounted for the unsaturation needed in the side group to fit the empirical formula of I and also for the single oxygen atom of I. The first possibility was eliminated by the finding that I contained no carbon methyl group. Arguments against the presence of a furan nucleus were that I did not absorb hydrogen over platinum or palladium catalysts, did not undergo the Diels–Alder reaction with maleic anhydride, and showed none of the acid sensitivity common to many furan types. It was obvious, therefore, that the furan ring, if actually present in I, had to be so located as to account for this marked lack of reactivity. In this connection it is known that attachment of an amino group to the α-position of the side chain on a furan ring (furfurylamine type) greatly stabilizes the ring.[9] Therefore, it was tentatively concluded that the unidentified side group consisted of a furfuryl substituent attached to a nitrogen atom.

The next question was the point of attachment of the presumed furfuryl side chain to the adenine nucleus. Since I had a pK_a value near 10 and was precipitated by silver ions from acid solution, the 9-position was unsubstituted. However, the 6-amino group was apparently not free, since acetylation was repeatedly unsuccessful and a Van Slyke determination carried out under conditions which give quantitative results with adenine[10] was essentially negative.

In the light of all the evidence listed above, it was concluded that I most probably was 6-furfurylaminopurine. Synthesis of a compound of this structure was, therefore, undertaken, first by means of direct reaction between adenine and furfuryl chloride. The crude product of this synthesis gave three ultraviolet quenching spots on a paper chromatogram; one of these had the R_F value of I and gave a positive Dische test. Subsequently it was determined that the eluate of this spot possessed distinct cell-division activity, but as no pure product was readily obtainable by this method a better synthesis was sought.

When the procedure of Elion, Burgi and Hitchings[11] for the synthesis of N^6-alkyl substituted adenines was carried out with 6-methylmercaptopurine and furfurylamine, a good yield of a crystalline product was obtained readily. This synthetic product, purified by recrystallization from absolute ethanol in the same manner as I, proved to be identical with the isolated substance, thus definitely establishing the structure of I as that of 6-furfurylaminopurine.

A characteristic physiological effect of this compound is to permit cytokinesis (*i.e.*, the partition of a cell into new cells) and thus to permit continuous growth of various plant tissues *in vitro*. For example, in tobacco pith tissue in the absence of I or other compounds with similar activity, mitosis may occur to some extent so that some cells with 2, 4 or even 8 nuclei may be formed without the occurrence of cell division.[12,13] Such tissue forms callus and has now been subcultured through five transfers on a medium containing 200 μg./l. of I. Pieces of this tissue which were being transferred to control media and thus deprived of the compound at each successive subculturing have stopped growing. Thus the compound must be present if cell division is to continue. Promotion of cell multiplication has been observed with as little as 1 μg./l. of I. Details of this and other striking effects on various plant growth phenomena such as the initiation and development of buds and roots, the stimulation of seedling growth, and the enhancement of seed germination will be presented elsewhere.

Because I specifically promotes cytokinesis even in exceedingly low concentrations, the name *kinetin* (pronounced *kine'-uh-tin*) has been proposed for it. It is evident that kinetin satisfies the requirement for only one of many essential growth factors and that even when this requirement is the limiting factor for cell division, kinetin may be replaced by any one of a number of substances with similar activity. For example, several active analogs of kine-

(5) J. S. Wall, *Anal. Chem.*, **25**, 950 (1953).

(6) Removal of two molecules of water from an adenine deoxypentoside, for example, would lead to the correct empirical formula, $C_{10}H_9N_5O$. However, no cell division activity was generated by autoclaving adenine deoxyriboside under conditions that were effective with DNA. Addendum: Success has since been reported by R. H. Hall and R. S. de Ropp, THIS JOURNAL, **77**, 6400 (1955).

(7) Z. Dische, *Proc. Soc. Expll. Biol. Med.*, **55**, 217 (1944).

(8) P. K. Stumpf, *J. Biol. Chem.*, **169**, 367 (1947).

(9) A. P. Dunlop and F. N. Peters, "The Furans," Reinhold Publ. Corp., New York, N. Y., 1953, p. 170, 680.

(10) D. W. Wilson, *J. Biol. Chem.*, **56**, 183 (1923).

(11) G. B. Elion, E. Burgi and G. H. Hitchings, THIS JOURNAL, **74**, 411 (1952).

(12) J. Naylor, G. Sander and F. Skoog, *Physiol. Plantarum*, **7**, 25 (1954).

(13) F. Skoog, Chapt. 8, "Chemical Regulation of Growth. In Dynamics of Growth Processes," E. G. Boell Ed., Princeton University Press, Princeton, N. J., 1954, p. 148.

tin have now been synthesized,[14] and diphenylurea is reported to be active.[15] Consequently, there appears to be a need for a class name to cover all such substances, and the term *kinin* (pronounced *kine'-in*) is hereby proposed for this purpose. Thus, kinetin is one specific kinin, as indoleacetic acid is one specific auxin. Certain other substances, such as adenine, indoleacetic acid and casein hydrolysate may potentiate or intensify the effect of kinins, but cannot be substituted for them. As knowledge of the complex process of cell division and of the role of kinins therein increases, a more exact definition will no doubt become possible.

The question of whether kinetin exists as such and functions as a kinin in nature, or whether a kinetin derivative or a totally different type of substance is mainly responsible for fulfilling this function in living organisms, must be left open at present. Certainly the kinins in coconut extracts differ from kinetin in being non-extractable with ether, but it is quite conceivable that a purine derivative of the kinetin type could be present in coconuts. While isolation of the kinin from yeast has not yet been completed, there is no doubt that commercial dried yeast extract does contain a substance very similar to and quite possibly identical with kinetin.

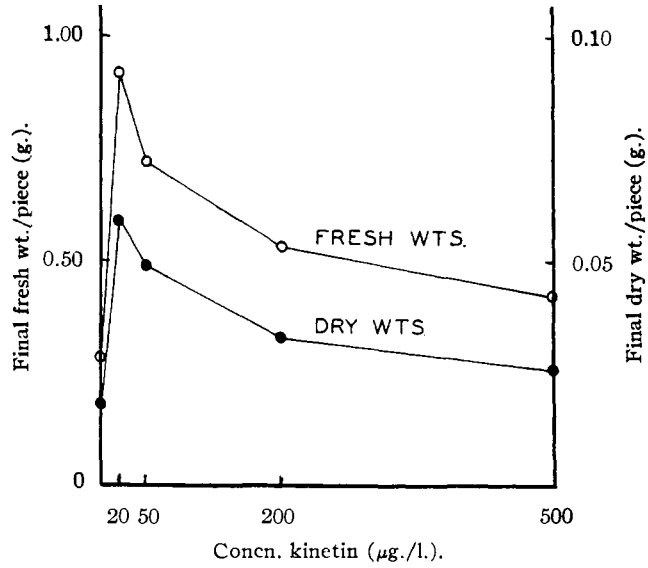

Fig. 1.—Effect of kinetin concentration on growth of tobacco callus tissue. Data, average weights per callus piece after 31 days of culture on White's medium plus 2 mg./l. IAA. 12 pieces per treatment, except only 9 pieces for treatment with 20 μg./l. kinetin. Initial fresh weight was 0.06 and dry weight 0.004 g. per piece.

Experimental[16]

Test Methods.—For the bioassay, callus tissue newly formed at the basal ends of tobacco stem segments was used.[17] These stem segments had been cultured for 3–4 weeks on a medium containing (mg./l.): $Ca(NO_3)_2$, 100; KNO_3, 80; $MgSO_4$, 35; KCl, 65; KH_2PO_4, 12; NH_4NO_3, 400; KI, 0.8; $MnSO_4$, 4.4; $ZnSO_4$, 1.5; H_3BO_3, 1.6; NaFe Sequestrene (sodium ferric ethylenediaminetetraacetate), 25; glycine, 2; thiamine, 0.1; nicotinic acid, 0.5; pyridoxine, 0.1; indoleacetic acid, 2; sucrose, 20,000; and agar, 10,000. The basal test medium was of this same composition and all media were adjusted to *p*H 6.0. Preparations to be tested for cell-division activity were made up in aqueous solutions and added to the basal medium of proper strength to give the final concentrations listed above. Usually the samples were tested at several different concentrations. Evaluations of various preparations were based upon increases of fresh and dry weights and even more upon visual estimates over a period of days of the relative growth of callus tissue pieces. Thus absolute values were not obtained for the efficiency of isolation steps nor for the potency of various concentrates. However, of all the isolation methods investigated, only those which obviously permitted good recovery and considerable purification of the active material were included as steps in the final isolation procedure. This approach seemed justified by the observations that total growth in terms of weight was greatly influenced by impurities in the preparations and that an inverse relationship existed between number and size of cells. Furthermore, exclusive use of cell counts as an index of activity would have slowed up the isolation work unduly. As indicated by a representative experiment in Fig. 1, kinetin at all levels tested increased both the fresh and dry weights of tobacco callus cultures, although the low concentrations were most effective. Promotion of cell division was verified in anatomical sections. The effect was particularly striking in cultures of excised tobacco pith tissue, of which controls without kinetin showed no cell divisions. It should be mentioned that not all the pith cells were stimulated to divide so that islands of new rapidly dividing cells were formed between the remaining mature cells. The uniformity of the response, *i.e.*, the number or proportion of mature cells which are stimulated to divide, increases with the kinetin concentration and with the concentration of auxin up to a point. With low concentrations the response may be restricted almost entirely to the basipetal end of the sections and is very comparable with the response elicited by pieces of vascular tissue placed in contact with the pith sections.[18]

Isolation Studies. A. Coconut.—Early in this work, extracts of numerous natural materials were found to promote cell division. Coconut meat, coconut milk, malt, yeast and tobacco leaves were active; beef extract and cow's milk were inactive. Coconut extracts were studied extensively.[3] Copra meal was found to be about 2.5 times richer in the cell-division stimulating factor than was fresh coconut milk.[4] This difference corresponds to the difference in moisture and fat content between the meal and fresh coconut meat and indicates that little or no activity is lost in the preparation of the meal. Two separate active components were obtained with R_F values of approximately 0.3 and 0.7, respectively, on paper chromatograms developed with a solvent system of 35% pyridine, 35% *n*-butanol and 30% water. Material from the latter zone was active at 4 p.p.m., and was completely free of ninhydrin-positive materials. Details of this work are given by Buyske.[4]

B. Yeast.—Later, preparations from brewer's yeast—but not primary yeast—were found greatly to promote cell division. Most of the active material in Difco yeast extract went into 95% ethanol during a Soxhlet extraction and stayed in the supernatant during a 24-hour cooling period. After removal of the ethanol from the supernatant, a small fraction of the activity could be precipitated from an acid (*p*H 3.8) solution with 5% aqueous silver nitrate. Usually however, first barium hydroxide was added to the supernatant and the resulting inactive precipitate removed before silver nitrate was added. Under these latter conditions, about one-half of the activity was found in the precipitate. After removal of the silver by hydrochloric acid, a small fraction of the active material went into a layer of di-

(14) F. S. Okumura, M. H. Von Saltza, F. M. Strong, C. O. Miller and Folke Skoog, abstracts of the 128th meeting of the American Chemical Society, Minneapolis, September, 1955.

(15) F. C. Steward, personal communication.

(16) Melting and boiling points uncorrected. Microanalyses by Micro Tech Laboratories, Skokie, Illinois, unless otherwise indicated. Ultraviolet spectra were determined on the Beckman Model DU or Cary recording spectrophotometer, and infrared spectra with the Baird Associates spectrophotometer.

(17) C. Miller and F. Skoog, *Amer. J. Bot.*, **40**, 768 (1953).

(18) J. R. Jablonski and F. Skoog, *Physiol. Plantarum*, **7**, 16 (1954).

ethyl ether. The amount removed—probably less than 10%, although this could not be determined accurately—was not increased by further extraction with ether. The ether-soluble material was chromatographed on Whatman No. 1 filter paper with an ascending solvent system consisting of *n*-butanol saturated either with water, acetic acid, formic acid or ammonium hydroxide, the acids or base affecting very little the movement of the active compound. With a *n*-butanol–20% formic acid solvent system the extreme R_F values for the active substance were 0.80 and 0.95. In all solvent systems, activity was consistently correlated with the presence of a compound having absorption maxima in ultraviolet light at 268 mμ in water and ethanol and at 275 mμ in aqueous sodium hydroxide (*p*H 12.0). Not enough active compound was obtained from yeast for complete characterization.

C. Nucleic Acids.—A ribonucleic acid preparation from yeast was inactive, but an old sample of DNA derived from herring sperm was extremely active. Furthermore, the active material in this sample was soluble in organic solvents. Fresh DNA samples were inactive, however, until they had been stirred into water and autoclaved. Dry heating at 100°, boiling in either acidic or basic solutions, or treatment with deoxyribonuclease were not effective. For activation, by autoclaving, the *p*H had to be below 6.0, with *p*H 4.2–4.5 apparently optimal, although activity was also obtained at *p*H 1.0. Autoclaving also produced great activity in an otherwise inactive preparation of calf thymus DNA. One sample of herring sperm DNA, which was inactive when first obtained, became quite active after standing for a year on the laboratory shelf. Presumably, then, the autoclaving brought about changes similar to those which may occur when DNA samples stand at room temperature for long periods of time.

The method used most successfully for the isolation of crystalline kinetin from DNA is outlined in Fig. 2. Optimal conditions for activation and isolation were not worked out in detail, however. In this case the DNA was autoclaved 60 minutes instead of 30 minutes.[1a] The yield was twice that obtained before, thus suggesting that active compound is formed during autoclaving. Diethyl ether was substituted for *n*-butanol in the extraction because it removed the kinetin as effectively and dissolved less inert solids.

500 g. DNA (from herring sperm)
↓
stirred into water (final vol. = 1500 ml., *p*H 4.2)
↓
autoclaved 60 min. at 15 lb./sq. in. (120°)
↓
cooled to room temp.; *p*H to 9.3 (NaOH)
↓
extracted 5 times with 1 l. ethyl ether each time
↓
ether layers combined and ether removed
↓
residue dissolved in 100 ml. 2 *N* HCl and put onto a 1.5 × 22 cm. Dowex 50 (H^+) column
↓
column washed with 100 ml. H_2O
↓
eluted with 2 *N* HCl at 0.8 ml./min., and collected eluate in 5-ml. fractions
↓
pooled eluates from fractions 65 to 170 put onto a 1.5 × 22 cm. Dowex 50 (H^+) column
↓
column washed with 100 ml. H_2O
↓
eluted with *N* NH_4OH; collection started when NH_3 and crystals appeared in eluate
↓
eluate adjusted to *p*H 9.3 (HCl) and placed at 4° for 1 day
↓
ppt. washed with water and then recrystallized from absolute ethanol after decolorizing with Darco G 60 charcoal
↓
Yield = 275 mg. crystalline kinetin

Fig. 2.—Flow sheet of procedure for isolation of kinetin from DNA.

The development of the present procedure was aided greatly by the observation that the activity from both yeast and DNA was correlated with an ultraviolet absorption maximum at 268 mμ in water or ethanol. Furthermore, the active DNA preparations gave a pink color on paper chromatograms sprayed with cysteine and sulfuric acid[19]; yeast concentrates were not tested in this manner. The pink product had the same absorption curve as that obtained by similar treatment of deoxyribose compounds.[8]

Analysis and Properties of Isolated Kinetin.—Repeated crystallizations from absolute ethanol of the product obtained as above gave slightly off-white platelets, m.p. 265–266° (dec., sealed tube). On the hot stage the substance sublimed at 220° without melting. Qualitative tests by the micro-method of Bennett, *et al.*,[20] were positive for nitrogen but negative for sulfur, halogens and phosphorus. For analysis a sample was dried at 100° *in vacuo* over calcium sulfate ("Drierite").

Anal. Calcd. for $C_{10}H_9N_5O$: C, 55.81; H, 4.22; N, 32.55; mol. wt., 215.2. Found (on three separate preparations): C, 56.06, 56.13, 56.16[21]; H, 4.09, 4.16, 4.12[21]; N, 32.55, 32.58, 32.0[21]; $-OCH_3$, nil[21]; $-NCH_3$, nil[21]; $-CCH_3$, nil.[21,22] Electrometric titration in 1:1 ethanol:water gave apparent pK_a values of 2.7 ± 0.2 and 9.9 ± 0.2; neut. equiv. (based on pK_{a_2}) 223 ± 15. This titration could not be carried out in water because I was too insoluble in the *p*H range 2–10. Estimation of pK_a values in water solution by the spectrophotometric method[23] was possible at 6×10^{-5} *M* concentration, and gave values of 3.8 ± 0.2 and 10.0 ± 0.2.

Kinetin showed no detectable optical rotation in 1 *N* sulfuric acid solution (*c* 1.8). The infrared spectrum is reproduced in Fig. 3. The ultraviolet spectrum showed a single band with the following inflection points

Solvent	λ_{max}, mμ	ϵ_{max}	λ_{min}, mμ	ϵ_{min}
Abs. ethanol	267.5	18,700	234.0	3200
1 *N* aq. HCl	274.0	17,000	235.5	3800
1 *N* aq. NaOH	273.5	17,600	241.0	4000
Aq. buffer, *p*H 6.4	267.0	18,800	233.5	3630

In addition the spectrum measured in aqueous alkali exhibited a shoulder at 283 mμ. A sharp isosbestic point at 271.5 mμ was observed when ultraviolet spectral curves were recorded for aqueous kinetin solutions varying in *p*H from 1.0 to 6.4. The substance gave an immediate, heavy, amorphous, white precipitate when a solution in 0.05 *N* sulfuric acid was treated with 5% aqueous silver nitrate solution. The Van Slyke determination was carried out as described by Wilson for adenine. After 1 hour shaking and 1 hour standing at 28° nitrogen evolution amounted to 35% of that required for one amino group.

Attempted Reactions with Kinetin. A. Micro-hydrogenation.—No hydrogen was absorbed when a solution of 13.7 mg. of kinetin in 5 ml. of 0.1 *N* hydrochloric acid was shaken for 2 hours under hydrogen with 9.7 mg. of a 10% palladium on charcoal catalyst. The experiment was repeated with Adams platinum oxide catalyst with the same result.

B. Acetylation.—A solution of 5 mg. of kinetin in 0.1 ml. of acetic anhydride was cooled in ice, *ca.* 0.01 ml. of concentrated sulfuric acid added, and the mixture kept overnight at 4°. The excess acetic anhydride was decomposed with water, the mixture evaporated to dryness, and the residue crystallized from ethanol. Unchanged kinetin, m.p. 265–266° (sealed tube) was the only product obtained. Acetylation was also attempted with acetic anhydride alone, acetic anhydride plus 85% phosphoric acid,[24] acetic anhydride plus 10% aqueous sodium hydroxide, acetyl chloride alone, and acetyl chloride plus pyridine, but in all cases only unchanged kinetin was recovered.

C. Condensation with Maleic Anhydride.—A solution of 10.3 mg. of kinetin and 5.8 mg. of maleic anhydride in *ca.* 0.1 ml. of dioxane was heated for 2 hours at 50–60°, then placed at 4° for several hours. A small amount of solid which separated from the solution was centrifuged off,

(19) J. G. Buchanan, *Nature*, **168**, 1091 (1951).
(20) E. L. Bennett, C. W. Gould, Jr., E. H. Swift and Carl Niemann, *Anal. Chem.*, **19**, 1035 (1947).
(21) Weiler and Strauss, Oxford, England.
(22) Huffman Microanalytical Laboratories, Wheatridge, Colorado.
(23) D. Shugar and J. J. Fox, *Biochim. Biophys. Acta*, **9**, 199 (1952).
(24) R. Adams and H. W. Stewart, THIS JOURNAL, **63**, 2860 (1941).

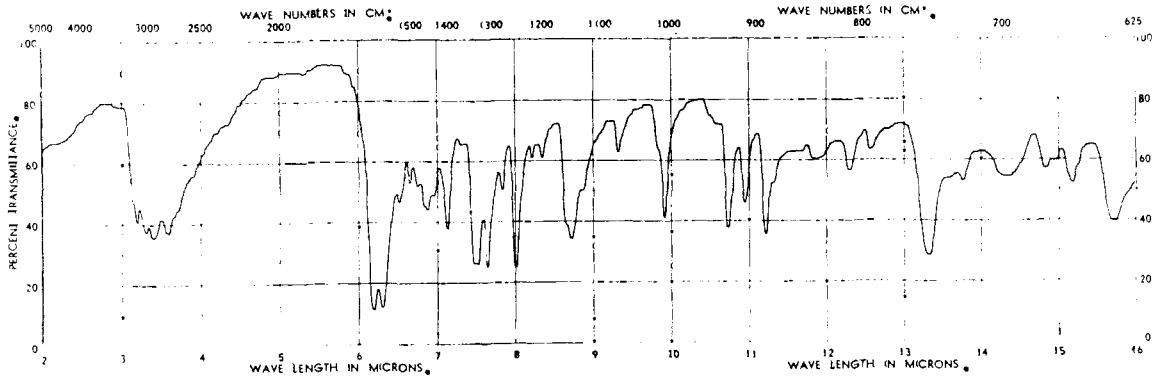

Fig. 3.—Infrared spectrum of kinetin isolated from DNA, taken in KBr pellet.

washed with dioxane and water, and dried, m.p. above 300°. The solution was evaporated to dryness, and the residue washed with alcohol and dried (main product). The m.p. of 255–257° was not depressed by admixture with kinetin.

Degradation Experiments.—A preliminary degradation trial was carried out by autoclaving 2 mg. of isolated kinetin in 2 *N* hydrochloric acid solution for 2 hours at 15 lb. pressure (120°). The acid was removed by evaporation, the residue taken up in water, the solution adjusted to *p*H 6.0 with sodium hydroxide, and a small portion chromatographed on Whatman No. 1 filter paper using water-saturated *n*-butanol as ascending solvent. A strong ultraviolet quenching spot at R_F 0.33 was observed, and a faint one at R_F 0.80. The former gave a negative Dische test[7,19] and when eluted with water showed absorption maxima at 260 mμ in water, 261 mμ in 2 *N* hydrochloric acid, and 268 mμ in 0.1 *N* sodium hydroxide. The position of these maxima and the R_F value of 0.33 corresponded exactly with those found when known adenine was chromatographed under the same conditions. Since the material in the faint spot at R_F 0.80 gave a positive Dische test and, when eluted, showed a maximum at 267 mμ in water, it was unchanged kinetin.

Another portion of kinetin, 21.5 mg. (0.1 mmole), was dissolved in 25 ml. of 1 *N* sulfuric acid. Refluxing this solution for one hour caused a change in the ultraviolet absorption spectrum which was calculated, on the basis of the spectra of known kinetin and adenine solutions, to correspond to breakdown of 10% or less of the original kinetin present. A control spectral study of an equimolar mixture of adenine and levulinic acid showed that the latter had no appreciable effect on the ultraviolet absorption spectrum of the former.

The same solution was then autoclaved for one hour at 15 lb. pressure (120°), and the ultraviolet spectrum again determined. The shift in position and extinction value of the maximum indicated approximately 40–60% destruction of the kinetin. After one more hour of autoclaving, destruction was assumed to be essentially complete, although the above paper chromatography results would indicate that traces of kinetin might survive this treatment. The mixture at this stage was pale yellow in color, and had a strong odor of levulinic acid.

On the basis of the above preliminary results, an attempt was made to degrade kinetin into adenine and levulinic acid and identify these products more positively. A solution of 21.5 mg. (0.1 mmole) of kinetin in 1 ml. of 2 *N* hydrochloric acid was placed in the extraction tube of a micro liquid–liquid continuous extractor and autoclaved at 15 lb. (120°) for two hours. The brown colored solution was then exhaustively extracted with ether during a five-hour period. The boiling flask of the extraction apparatus contained 50 ml. of ether plus 1.5 ml. of concentrated sulfuric acid and 23 mg. of 2,4-dinitrophenylhydrazine. After the extraction was complete the ether extract was separated from the sulfuric acid layer, evaporated to dryness, and yielded a few mg. of amorphous orange-yellow residue. Attempts to crystallize this product failed. A sample was chromatographed on paper in a *n*-butanol:3% aqueous ammonia system and gave a single yellow spot with the same color and R_F value (0.64) as that of authentic levulinic acid 2,4-dinitrophenylhydrazone in this system.

The *p*H of the ether-extracted aqueous hydrolyzate was raised to 5 with sodium hydroxide, whereupon a black precipitate formed. This was centrifuged off, the supernatant evaporated to 10 ml., and a saturated solution of picric acid in ethanol added. A yellow semicrystalline precipitate was obtained, m.p. 292–294°, when taken as recommended by Vickery.[25] The yield was 11.5 mg., 32%. Mixed with known adenine picrate, m.p. 298–299°, the m.p. was 291–293°.

A 2.0-mg. portion of the above crude picrate was converted to the chloride by dissolving in hot water, stirring with Dowex-1 (Cl^- form), and filtering.[26] The filtrate was evaporated to 1 ml. and 1 ml. of 3 *N* hydrochloric acid added. This solution was then poured onto a 1.2 × 55 cm. column of 200–400 mesh Dowex-50, 12% cross-linked cation-exchange resin prepared as described by Wall.[5] The column was then washed successively with the following volumes and normalities of hydrochloric acid: 91 ml. of 1.5 *N*, 315 ml. of 2.5 *N*, 70 ml. of 4.0 *N*, and 147 ml. of 6.0 *N*. The eluate was collected in 7-ml. fractions and the 262 mμ absorption of each fraction determined. The main peak (85% of the total 262 mμ absorption) came off with 6 *N* acid, as does adenine,[5] and the complete ultraviolet absorption curve of the material in this peak was indistinguishable from that of adenine.

Synthesis of 6-Furfurylaminopurine. A. From Adenine.—First attempts were made to condense adenine with furfural with the intention of reducing the Schiff base to form I. However, no definite condensation product could be obtained.

Direct alkylation of adenine with furfuryl chloride was somewhat more successful. A mixture of 0.5 g. of adenine (free base), 0.7 g. of freshly redistilled furfuryl chloride and 0.5 g. of sodium bicarbonate was heated on a hot plate. When the temperature reached 90°, the mixture suddenly foamed and the temperature rose to 110°. After heating at 110° for five minutes longer, the reddish brown mixture was cooled, stirred up several times with ether, and the insoluble residue dissolved in water. This aqueous solution, when chromatographed on paper with water-saturated *n*-butanol as the ascending solvent system, showed ultraviolet-quenching bands with R_F values of 0.97, 0.80 and 0.33. The materials at R_F 0.97 and 0.80 gave pink colors when sprayed with the cysteine–H_2SO_4 reagent. Only the material at R_F 0.80 moved to the same region as kinetin when water alone was used as the solvent, and it had an absorption maximum at 268 mμ in absolute ethanol, as has kinetin. Furthermore, this material when eluted and tested showed the same type activity in cell division as does kinetin.

B. From 6-Methylmercaptopurine.—A mixture of 4.15 g. (0.025 mole) of 6-methylmercaptopurine,[11] m.p. 207–209° and 9.6 g. (0.1 mole) of freshly redistilled furfurylamine was heated at 115–120° for 9 hours, then placed at 4° for several hours. Two volumes of acetone were added to the brownish colored reaction mixture, and the solid product filtered off and washed with acetone. The crude, tan-colored, crystalline solid was recrystallized from absolute ethanol, using Darco G-60 for decolorization. Colorless

(25) H. B. Vickery and C. S. Leavenworth, *J. Biol. Chem.*, **63**, 579 (1925).

(26) J. Davoll and B. A. Lowy, THIS JOURNAL, **73**, 1655 (1951).

platelets, m.p. 266–267°, were obtained in the amount of 4.45 g. (82.8%). A mixed melting point with isolated kinetin gave no depression.

The synthetic product proved indistinguishable from isolated kinetin when examined in the following ways: paper chromatography in various solvent systems as described above, color test with cysteine and sulfuric acid,[19] ultraviolet spectrum in neutral, acidic and alkaline solutions, and infrared spectrum.

Furthermore, in the following biological tests, in which effects of kinetin have been observed, no differences could be detected between the activities of the isolated and synthetic products: Promotion of cell division in tobacco callus and other plant tissue cultures; induction (in combination with auxin, IAA) of cell division and continuous proliferation of tobacco pith tissue *in vitro;* promotion (in combination with adenine) of bud initiation and development in tobacco stem segment cultures; promotion (in combination with IAA) of root initiation and development on cuttings; promotion or inhibition, depending on the concentration, of seedling growth in nutrient solution cultures; stimulation of lettuce seed germination and the inhibition of cell enlargement in pea stem section tests.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF ROCHESTER]

The Synthesis of Morphine

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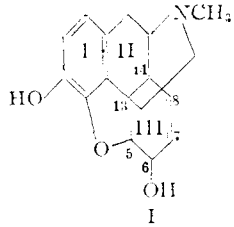
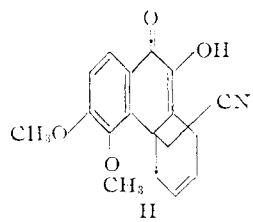
The completion of the first synthesis of morphine is described.

Morphine, the principal alkaloid of opium and the substance primarily responsible for its physiological effect, has attracted the attention of chemists for over one hundred and fifty years. Alkaloid chemistry may correctly be considered to have originated with Sertürner's[1] isolation of morphine from opium and his recognition of its basic character. Early analyses by Liebig, Dumas, Pelletier, Raoult and others serve to illustrate its intimate association with the development of the infant science of chemistry. An equally illustrious group of men contributed to structural studies during a later period, and in our time these structural studies, initiated by Hesse, Vongerichten, Knorr, Pschorr and Freund and carried on brilliantly by Speyer, von Braun, Wieland, Robinson and Schöpf, have culminated in Robinson's[2] proposal in 1925 of the correct structure for morphine (I). In this paper[3] we describe the completion of the first synthesis of morphine and therewith a complete confirmation of the Robinson formula.



I



II

The synthesis and proof of structure of 3,4-dimethoxy - 9,10 - dioxo - 13 - cyanomethyl - 5,8,9,-10,13,14-hexahydrophenanthrene (II) were described in an earlier paper.[4] This adduct on hydrogenation over copper chromite under relatively mild conditions (130°, 27 atm. of hydrogen) undergoes reductive cyclization in the manner observed earlier[5] in a model series to give the keto-lactam III in 50% yield. The weak basicity of this substance and its prominent bands at 5.93 and 6.03 μ in the infrared and at 281 mμ (log ϵ 4.16) in the ultraviolet are consistent with this formulation. The corresponding 6-chloro adduct can likewise be converted into IV, but the poor yields in this reaction and in the preceding Diels–Alder reactions[4] with chloroprene and with 2-ethoxybutadiene discouraged further work with these 6-substituted derivatives, and we were forced to rely on a later introduction of oxygen at C_6. The course of this reductive cyclization, which leads to a tetracyclic carbon–nitrogen skeleton stereoisomeric with that of the morphine alkaloids, is far from clear.[6]

The carbonyl group at C_{10} of III is readily removed by the Wolff–Kishner method. Under the conditions recommended by Huang-Minlon[7] extensive demethylation takes place and the yields of the lactam V after remethylation do not exceed 56%. We find, however, that this reduction

(1) F. W. Sertürner, *Trommsdorf's Journal der Pharmazie*, **13**, 1, 234 (1805); *Ann. chim. phys.*, [2] **5**, 21 (1817).

(2) J. M. Gulland and R. Robinson, *Mem. Proc. Manchester Lit. Phil. Soc.*, **69**, 79 (1925).

(3) Preliminary accounts of this work have appeared, in THIS JOURNAL, **72**, 4839 (1950), and **74**, 1109 (1952).

(4) M. Gates, *ibid.*, **72**, 228 (1950).

(5) M. Gates, R. B. Woodward, W. F. Newhall and R. Künzli, *ibid.*, **72**, 1141 (1950).

(6) It may be pointed out that if hydrogen is added to the 9,14-double bond of the enolic adduct on the side opposite the cyanomethyl group at C_{13}, the observed *trans* (*vide infra*) ring juncture at C_{13}–C_{14} results. Alkylation of the nitrile group by C_8 would then yield the lactam, either by a process analogous to that observed by Ritter (J. J. Ritter and P. P. Minieri, *ibid.*, **70**, 4045 (1948), and later papers in this series) or by rearrangement of an iminoether formed between the nitrile and the hydroxyl at C_8. It is a pleasure to acknowledge a number of very helpful discussions of this reaction with Professor R. B. Woodward, who first correctly inferred the structure of the keto-lactam resulting from this reductive cyclization in the model series.[5]

(7) Huang-Minlon, *ibid.*, **68**, 2487 (1946).